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13. ABSTRACT *(Maximum 200 Words)*

This study addressed the interaction of environmental and genetic factors in the etiology of breast cancer. Polycyclic aromatic hydrocarbons (PAHs) are metabolically activated to the ultimate carcinogen by the cytochrome P450 isozymes CYP1A1 and CYP1B1. We have investigated the hypothesis that increased expression of *CYP1B1* and/or *CYP1A1* in breast tissue represents a risk factor for breast cancer. We have determined expression of both *CYP1A1* and *CYP1B1* genes in histologically normal breast tissue specimens from 36 breast cancer patients and 39 cancer-free individuals. Using a semiquantitative RT-PCR assay we measured *CYP1A1* and *CYP1B1* expression relative to the constantly expressed β-actin gene. We found a large variation in expression of both genes between individuals, and for most samples CYP1B1 transcript levels were 2-7 times higher than CYP1A1. The mean CYP1B1 transcript level in normal breast tissue was 70% higher in breast cancer patients compared with cancer-free controls ($p = 0.0473$). Genetic polymorphisms in the *CYP1A1* and *CYP1B1* genes were also analyzed, but no difference in variant frequencies between cases and controls was found. We conclude that CYP1B1 is an important PAH-activating enzyme in breast and that high levels can represent a risk factor for breast cancer.

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FOREWORD

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Regine Goth Goldstein 3/28/2002

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Interindividual Differences in Metabolism of Carcinogens as a Risk Factor for Breast Cancer

Introduction

Numerous studies indicate that exposure to polycyclic aromatic hydrocarbons (PAHs) increases the risk of developing certain types of human cancers (1). The major pathway by which ingested or inhaled PAHs are metabolized, is the stepwise oxidative activation by the cytochrome P450 isozymes, CYP1A1 and CYP1B1, followed by detoxification by phase II enzymes (2). The highly reactive intermediates formed by CYP1A1 or CYP1B1 can bind to DNA, and the resulting DNA adducts can cause a mutation that if in a relevant gene could initiate cancer. Expression of both CYP1A1 and CYP1B1 is highly inducible by PAHs and other environmental toxins, such as dioxin (3). Whereas CYP1A1 has been studied extensively for over 25 years, much less is known about CYP1B1, one of the newest members of the P450 family. There is considerable evidence now that CYP1B1 could be a key enzyme in the activation of carcinogens in the breast and therefore play a role in the development of breast cancer. The CYP1B1 gene is highly expressed in human breast tissue, but not in liver, which has been considered the major site for metabolism of xenobiotic compounds (4). Experiments with recombinant human enzymes showed that CYP1B1 is the principal enzyme in catalyzing oxidation of benzo[a]pyrene to the diolepoxide, being ten times more efficient than CYP1A1(5). When investigating 7,12-dimethylbenz(a)anthracene-induced lymphomas in mice, the frequency of lymphomas was reduced to one tenth in CYP1B1 null mice compared to wild-type mice (6) and CYP1B1 null mice were protected from the bone marrow cytotoxic effects (7), indicating that CYP1B1 is critical for carcinogenesis by certain PAHs and that extrahepatic metabolism is important in determining susceptibility to PAHs. CYP1A1 and CYP1B1 are also involved in estrogen hydroxylation, but they differ in the position of hydroxylation; whereas CYP1A1 acts at the C-2 position, CYP1B1 acts at the C-4 position leading to formation of the potentially carcinogenic 4-hydroxy estradiol (8).

We have tested the hypothesis that individuals with higher levels of CYP1B1 are at a higher risk for breast cancer because they produce higher amounts of ultimate carcinogen. The expression level of *CYP1B1* was determined in a collection of histologically normal breast tissue samples from reduction mammoplasties and from mastectomy patients and *CYP1B1* expression was compared in specimen from cancer patients and healthy controls to establish if breast cancer patients have an increased level of the enzyme.

Body of Final Report

Interindividual variation in carcinogen metabolism has been recognized as an important determinant of susceptibility to various cancers. We are testing the hypothesis that the level and activity of enzymes with the capacity to activate environmental carcinogens in

the breast represent a risk factor for breast cancer, and specifically that individuals with higher levels of CYP1B1 are at a higher risk for breast cancer because they produce higher amounts of ultimate carcinogens. A genetic polymorphism in the structural gene of an enzyme can result in an alteration of the activity of the enzyme and represents a lifelong change. The amount of an enzyme, in case of *CYP1B1* regulated at the transcriptional level, can depend on genetic and environmental factors. Hormones, diet, smoking habits, and exposure to environmental contaminants, such as pesticides or dioxin –like compounds, can modify expression of *CYP1B1* in an individual by acting as inducers or repressors. Whereas a given genetic polymorphism capture only a fraction of the enzyme variability in at risk individuals, expression is the result of multiple factors, and when measuring expression of *CYP1B1* one captures all possible modifying factors.
A collection of histologically normal breast tissue specimens from mastectomy patients and from reduction mammoplasties has been analyzed both for expression of the *CYP1A1* and *CYP1B1* genes and for known polymorphisms in these genes.

Task 1. To compare expression of *CYP1B1* in healthy individuals and breast cancer patients

A limited number of specimens from a breast tissue bank established by Dr. Martha Stampfer were available for analysis at the beginning of this study. During the period of this study, specimens from a second tissue bank became available. This tissue bank had been established by Peralta Cancer Center in 1981-1988 and was maintained by Aeron Biotechnology Inc. Specimens from this second tissue bank were of lower quality, in that about a quarter of the samples gave no amplifiable RNA. Only histologically normal breast tissue specimens (as determined by pathology report) were used for expression studies. The specimens had been dissected and isolated from adipose and connective tissue, so that only epithelial material was stored frozen as organoids. All tissue obtained from surgery of one breast was processed as one sample, so that there are no specimens from different locations in the breast available. Of the specimens that provided amplifiable RNA, the control group consisted of 39 reduction mammoplasty specimens. The case group consisted of 32 peripheral non-tumor tissue specimens obtained from mastectomy patients and 4 tissue specimens contralateral to the tumor.

a. RT-PCR assay for quantitation of CYP1B1 transcripts
General approach to method: Expression of *CYP1B1* was measured in parallel to *CYP1A1* by a semiquantitative method developed in our lab for *CYP1A1* (9). cDNA obtained from the breast epithelial cells was serially diluted, then amplified three times: first with CYP1B1 primers (10), then with CYP1A1 primers (11), and finally with β-actin primers (12). All primer sets are designed to span an intron, thus excluding amplification of any contaminating genomic DNA and generated products of 545 bp for CYP1B1, 320 bp for CYP1A1, and 273 bp for β-actin. As PCR is quantitative only in the exponential phase of product accumulation, PCR conditions and cycle numbers for each target were optimized separately for each primer pair. The cycle numbers for each target were

chosen to give appropriate sensitivity while avoiding PCR plateau phase. The CYP1B1 RT-PCR assay was optimized to give the same amplification efficiency as the CYP1A1 reaction. After amplification, the products were mixed together before electrophoresis on a 10% native polyacrylamide gel. The gel was stained with SYBR Gold nucleic acid stain and scanned without destaining on a Molecular Dynamics STORM 860 laser scanner. The fluorescent signal for each band was quantitated using ImageQuant software after background correction included in the software. CYP1A1 and CYPIB1 levels are expressed as a ratio to β-actin values. For details of assay conditions and data analysis see *Appendix 2*. Specimens were analyzed in groups of 10 together with a positive control to assure that measurements were in the previously measured range.

Reliability of method: The reliability of the semiquantitative assay to detect *CYP1B1* expression was tested in a number of ways. After all the specimens had been analyzed, 15% of the cDNA was re-amplified and the CYP1B1 and CYP1A1 levels were found to repeat within two-fold. Also, for one reduction mammoplasty specimen for which several samples were available *CYP1B1* expression was measured in triplicate samples that had been frozen down separately. The results showed a good agreement between the three samples with less than 3% variance in CYP1B1 expression.
It was also determined how similar *CYP1B1* expression is in specimens from left and right breast to address the question of local variation of gene expression in breast tissue. The tissue specimens from left and right breast had been processed as separate samples. For three pairs tested, CYP1B1 transcript levels varied by less than 15% among each pair (CYP1B1/β-actin in breast tissue from left versus right: 0.97, 1.18; 3.12, 2.69; 1.17, 1.00). However when comparing *CYP1B1* expression in breast cells and skin cells of a mastectomy patient, a 30-fold difference in CYP1B1 transcript levels was found (CYP1B1/β-actin in breast tissue 29.2 in skin 0.92), indicating a large variation in *CYP1B1* expression between different tissues in one individual.

b.- d. Expression of the *CYP1B1* in breast tissue specimens
We determined expression of the *CYP1B1* gene and in parallel the *CYP1A1* gene in a total of 75 specimens of non-tumor breast tissue from 36 mastectomy patients and from 39 reduction mammoplasties.

Comparison of CYP1B1 and *CYP1A1 expression:* There was a more than 300-fold variation between individuals in the CYP1B1/β-actin ratio (range 0.43 to 132.50) and a more than 1000-fold variation in the CYP1A1/β-actin ratio (range 0.06 to 66.81). *CYP1B1* was expressed at lower levels than *CYP1A1* in only one specimen, at similar levels as CYP1A1 in 13 specimens (ratio 0.8-1.4), and at higher levels than *CYP1A1* (ratio 2 - 82) in the remaining 61 specimens (81% of the sample). The mean CYP1B1 to CYP1A1 ratio was 7.97 and the median ratio was 3.9. The mean ratio did not differ between cases (mean = 7.84, SD = 13.01) and controls (mean = 8.11, SD = 10.58). Seven specimen donors (3 cases and 4 controls) had a CYP1B1/CYP1A1 ratio $\geq$ 20; all were under 50 years of age.

Comparison of CYP1B1 expression in breast cancer patients and controls: Whereas the means in CYP1A1 values between the study groups were not statistical different ($p = 0.3005$), the difference in CYP1B1 values between the study groups was statistically significant ($p = 0.0473$) with more specimens with high *CYP1B1* expression among breast cancer patients than among healthy controls (Table 1). *CYP1A1* expression in breast tissue has been found previously to be independent of age (9). *CYP1B1* expression does not appear to be linearly related to age ($r = 0.071$, $p = 0.5549$). However, when the data were split into two age groups (< 50 and ≥ 50), which roughly correspond to menopausal status, the range of CYP1B1 values was noticeably more restricted in controls over 50, possibly indicating that endogenous estrogen levels might influence *CYP1B1* expression.

Table 1: Comparison of *CYP1A1* and *CYP1B1* expression in non-tumor breast tissue of breast cancer patients and healthy controls

CYP	Summary Statistic	Cases	Controls	p-value
CYP1A1	arithmetic mean (SD)	8.39* (11.57)	6.00* (8.12)	0.3005
	geometric mean (SD)	4.18 (3.82)	2.40 (5.03)	0.1111
	median	5.24	3.81	N.A.
	range	0.24 - 66.81	0.06 - 38.35	N.A.
CYP1B1	arithmetic mean (SD)	29.53(32.60)	17.48 (17.42)	0.0473
	geometric mean (SD)	15.82 (3.41)	10.18 (3.37)	0.1225
	median	16.13	10.48	N.A.
	range	1.52 - 132.5	0.43 - 79.3	N.A.

* These values reflect repeat measurements of CYP1A1 expression simultaneously with CYP1B1 expression of 56 specimens previously analyzed in ref. 9 (32 controls and 24 cases).

CYP1B1 and CYP1A1 expression in non-tumor versus tumor tissue: For 5 cases, non-tumor tissue, tumor tissue, and/or metastasis to the lymph nodes from the same individual were available. We compared *CYP1B1* expression in tumor tissue versus normal tissue to

evaluate whether higher CYP1B1 levels might be related to the disease process. The results were ambiguous (Table 2). For one individual, non-tumor tissue had higher *CYP1B1* expression than tumor tissue. For 3 individuals, expression was higher in tumor than non-tumor tissue. In one individual, where 2 different tumors and 2 metastases to the lymph node were available, *CYP1B1* expression in non-tumor tissue, 1 tumor, and 1

Table 2: Comparison of *CYP1B1* and *CYP1A1* expression in non-tumor versus tumor tissue for five individuals

ID	Specimen	CYP1B1/β-actin (relative difference)		CYP1A1/β-actin (relative difference)	
380	peripheral/non-tumor	4.46	(1.00)	0.74	(1.00)
	tumor in left breast	11.45	(2.57)	0.77	(1.04)
	tumor in right breast	4.45	(1.00)	0.45	(0.61)
	met* to left ln*	4.83	(1.08)	0.35	(0.47)
	met* to right ln*	41.87	(9.39)	6.21	(8.39)
383	peripheral/non-tumor	10.41	(1.00)	7.56	(1.00)
	tumor	6.31	(0.61)	1.33	(0.18)
407	peripheral/non-tumor	1.65	(1.00)	0.25	(1.00)
	tumor	6.92	(4.19)	1.73	(6.92)
412	peripheral/non-tumor	15.45	(1.00)	4.45	(1.00)
	tumor	66.88	(4.33)	10.80	(2.43)
426	peripheral/non-tumor	4.60	(1.00)	5.75	(1.00)
	met* to ln*	71.40	(15.52)	9.51	(1.65)

* met – metastasis, ln – lymph node

metastasis was comparable, but in 1 tumor and the other metastasis, *CYP1B1* expression was increased 3-fold and 9-fold, respectively. CYP1A1 expression varied less between non-tumor and tumor tissue.

Overall, these studies on *CYP1B1 and CYP1A1* expression indicate that CYP1B1 is a major enzyme for PAH metabolism in the breast and might therefore have a role in PAH-carcinogenesis. The results support our hypothesis that individuals with higher levels of CYP1B1 are at a higher risk for breast cancer.

Task 2. To determine *CYP1A1* and *CYP1B1* genotype in cases and controls

Besides the level of expression, genetic polymorphisms in the *CYP1B1* or *CYP1A1* gene can also alter the metabolic efficiency of these enzymes and thereby alter the activation step of PAHs to the ultimate carcinogen.

a. *CYP1B1* polymorphism

The original goal of this task had been to try to identify *CYP1B1* polymorphisms from specimens that have high CYP1B1, but low CYP1A1 transcript levels. However, since initiating this project several polymorphisms in the *CYP1B1* gene have been reported (13-15). Some DNA sequence changes that result in frameshift and missense mutations, have been associated with the development of primary congenital glaucoma, an autosomal recessive disease (13, 14), indicating that CYP1B1 has a physiological function in addition to PAH bioactivation. Based on the extensive DNA sequencing analysis of the *CYP1B1* gene in over 100 individuals in the published reports, it seems unlikely that there are additional polymorphisms in the *CYP1B1* coding region. Therefore we modified the original task slightly and analyzed our specimens for the known *CYP1B1* polymorphisms. Bailey et al. (15) described two common polymorphisms that result in amino acid changes, a Val to Leu change at codon 432 (m1) and a less common Asn to Ser change at codon 453 (m2). Both variants occur in exon 3 (which contains the heme binding domain of the enzyme) and result in altered enzyme activity (16, 17). Our collection of breast tissue specimens was analyzed for these polymorphisms according to the procedure described by Bailey et al. (15). We determined whether these polymorphisms were present in a total of 161 individuals: 97 specimens from breast cancer cases and 64 specimens from controls. The number of samples used for genotype analysis is higher than the number of samples, which were analyzed for gene expression, because only epithelial cell isolates of non-tumor tissue were used for expression studies, whereas genotype analysis could be performed on any cells from an individual.

Genetic linkage of two CYP1B1 polymorphisms: In 161 samples analyzed, no 453 Ser genotype was found in a 432 Val background (Table 3). We conclude that the *CYP1B1* 453 Ser (m2) polymorphism is linked to the 432 Leu genotype, that is, the Asn to Ser mutation originated in an individual with a 432 Leu genotype.

Table 3: Linkage of *CYP1B1* 453 Ser genotype to the 432 Leu genotype

		432 Variant		
		Val/Val	Val/Leu	Leu/Leu
453 Variant	Asn/Asn	42	46	22
	Asn/Ser	-	27	23
	Ser/Ser	-	-	1

Genotype frequency in cases and controls: The distribution among the m1 polymorphisms did not differ for cases and controls. Likewise, the distribution among the m2 polymorphisms did not differ for cases and controls (Table 4). However, the current sample size is small, and much larger numbers will be required to determine if the variant genotypes occur at different frequency in cases and controls.

Table 4: *CYP1B1* m1 and m2 genotypes in breast cancer cases and controls

CYP1B1 Genotypes	Cases (n = 97)	Controls (n = 64)
m1 (codon 432)		
Val/Val	25 (26%)	17 (27%)
Val/Leu	26 (43%)	18 (48%)
Leu/Leu	30 (31%)	16 (25%)
m2 (codon 453)		
Asn/Asn	66 (68%)	44 (69%)
Asn/Ser	67 (31%)	20 (31%)
Ser/Ser	1 (1%)	0

The *CYP1B1* genetic variants studied here have been shown to have an increased estrogen hydroxylation activity compared to the wild type genotypes (16, 17). Given the carcinogenic and estrogenic potential of 4-hydroxy estradiol, the inheritance of variant *CYP1B1* genotypes could contribute to interindividual differences in breast cancer risk. The role of these polymorphisms as risk factors for cancer is not yet clear. Two studies on *CYP1B1* polymorphism and breast cancer risk conducted in different ethnic groups gave conflicting results (15, 18). A recent study found that the *CYP1B1* variant with increased activity for 4-hydroxyestradiol formation is a susceptibility factor for ovarian

cancer (21).

b. *CYP1A1* polymorphism

Four polymorphisms consisting of single base changes have been identified in the *CYP1A1* gene (20) and two of the polymorphic variants (MspI and Ile-Val) have been associated with increased risk of lung cancer in Japanese (21), though it is unclear whether these polymorphisms affect the inducibility and activity of CYP1A1 (22).

To investigate to what extent the *CYP1A1* genotype modifies *CYP1A1* expression, the *CYP1A1* genotype of all specimens was determined using PCR/RFLP analysis as described in (9), Appendix 1. A total of 8 MspI and Ile-Val variants were detected in the 58 samples, 7 in the cancer free group and one in the patient group. When all *CYP1A1* values were ranked, the *CYP1A1* variants were distributed between the lowest and highest expression values with all heterozygote variants and one of the two homozygote variant having *CYP1A1* values below the mean *CYP1A1* values. These findings indicate that the *CYP1A1* polymorphism has at most a minor role in determining the *CYP1A1* expression level.

c. *AHR* polymorphism

Two recent reports investigated polymorphisms in the human aryl hydrocarbon receptor in individuals with low *CYP1A1* inducibility (23) and in others with high *CYP1A1* inducibility (24). Some previously unreported polymorphisms were identified, but in spite of extremely labor intensive sequencing work on many individuals, none of these polymorphisms related to the phenotypes of low or high *CYP1A1* inducibility. In light of these large negative studies this task was not further pursued.

d. Genotype of high expression phenotypes

When *CYP1B1* expression in different *CYP1B1* genotypes was compared, it appeared, that *CYP1B1* expression is higher in cases having the wild type (Val 432/Asn 453) genotype compared with specimens having the variant (Leu 432/ Ser 453) genotypes. In controls, the pattern was less clear. These results were unexpected because the polymorphisms are located in the structural gene and therefore are known to affect the enzyme activity. However, it is unclear how these sequence changes might affect gene expression. The data should be considered preliminary due to the small number of specimens analyzed to date.

Table 5: *CYP1B1* expression in different genotype groups

CYP1B1 genotype		*CYP1B1* expression (relative to ß-actin)					
		Cases			Controls		
m1	m2	n	mean	range	n	mean	range
Val/Val	Asn/Asn	6	56.4	2.2-133	10	22.6	0.5-61
Val/Leu	Asn/Asn	8	35.7	8.2-129	9	10.9	3.9-32
Leu/Leu	Asn/Asn	6	33.5	4.5-71	2	33.4	19.8-47
Val/Leu	Asn/Ser	5	19.2	2.4-29	9	11.5	0.4-41
Leu/Leu	Asn/Ser	4	8.2	1.5-17	3	13.3	4.6-26

A simultaneous high *CYP1A1* and *CYP1B1* expression phenotypes could be indicative of environmental exposure to agents that induce *CYP1A1* and *CYP1B1* through the AHR pathway, such as PAHs or dioxin-like compounds. When the >75% expression values for *CYP1A1* (>9.4) and *CYP1B1* (>32) were compared, 12 specimens showed high values for both *CYP1A1* and *CYP1B1*. Of these 4 were from the control group, and 8 were from the case group. In the absence of any exposure information, these data are preliminary. But this might represent an approach for the future to evaluate the effects from environmental exposure.

Future goals

In the completed study, the sample size was quite small and not matched for cases and controls. Only the age and disease status of the specimen donors were known. No information was available on donors' race, lifestyle, smoking habits, or other potential confounding factors. We have now obtained funding from USAMRMC to further investigate the role of *CYP1B1* in breast cancer. *CYP1B1* expression will be measured in a matched case control study, in which PAH exposure will be estimated by questionnaire, and DNA adducts will be determined as biomarkers of effect.

Key Research Accomplishments

- A sensitive and reliable semiquantitative RT-PCR assay was developed to determine simultaneously expression of *CYP1B1* and *CYP1A1*.
- We found a 300-fold variation in expression of *CYP1B1* in 75 non-tumor breast tissue samples.
- *CYP1B1* was expressed at higher levels than *CYP1A1* in most samples, indicating that it is an important PAH-activating enzyme in breast.
- More breast cancer cases than controls showed high *CYP1B1* expression and this difference was statistically significant.
- The two *CYP1B1* polymorphisms 432 (Val/Leu) and 453 (Asn/Ser) did not differ between cases and controls.
- The 453 Ser variant genotype is strictly linked to the 432 Leu variant genotype.
- *CYP1B1* expression appeared higher in specimens with the wild type (Val 432/Asn 453) genotype compared with the variant (Leu 432/ Ser 453) genotypes.
- The two common *CYP1A1* variant genotype were not associated with high *CYP1A1* expression.

Reportable Outcomes

Peer reviewed publication:
Regine Goth-Goldstein, Christine A. Erdmann, Martha R. Stampfer, Marion L. Russell, Interindividual Variation in CYP1A1 Expression in Breast Tissue and the Role of Genetic Polymorphism, Carcinogenesis 21: 2119-2122, 2000.

Regine Goth-Goldstein, Christine A. Erdmann, Marion L. Russell, Cytochrome P4501B1 expression in normal breast tissue. Submitted to Mol.Carcinogenesis.

Abstracts
Regine Goth-Goldstein, Christine A. Erdmann, Marion L. Russell, 'CYP1B1 expression in normal human breast tissue specimen' Proc. Am. Ass. Cancer Res.41 # 807 (2000).

Regine Goth-Goldstein, Christine A. Erdmann, Marion L. Russell, '*CYP1B1* expression as a risk factor for breast cancer'. Environ. Mol. Mutagenesis 37, Suppl. 32, # 68, (2001).

Presentations:
Invited talk on 'Metabolism of Environmental Chemicals as Breast Cancer Risk' at the California Breast Cancer Research Symposium, 9/18/1999 in Los Angeles.

Poster presentation at AACR 4/2000, San Francisco 'CYP1B1 expression in normal human breast tissue specimen' by Regine Goth-Goldstein, Christine A. Erdmann, Marion L. Russell .

Poster presentation at a meeting on 'New Frontiers in Women's Health Research' at the UC Davis Cancer Center, 4/00, 'Variation in metabolism of carcinogens as a risk factor for breast cancer' by Regine Goth-Goldstein, Christine A. Erdmann, Marion L. Russell.

Poster presentation at the DoD-sponsored meeting 'Era of Hope' in Atlanta in June 2000, 'Interindividual Variation in Metabolism of Carcinogens as a Risk Factor for Breast Cancer' by Regine Goth-Goldstein, Christine A. Erdmann, Marion L. Russell.

Poster presentation at the Annual Environmental Mutagen Society Meeting 3/01, San Diego, '*CYP1B1* expression as a risk factor for breast cancer' by Regine Goth-Goldstein, Christine A. Erdmann, Marion L. Russell.

Poster presentation at the EMS meeting 'Breast Cancer & Environmental Mutagens', 9/2001, Research Triangle Park, 'Polymorphism in CYP1B1 and breast cancer risk' by Elise Fairbain, Kacee Fujinami, Joeli Marrera, Marion L. Russell, Christine A. Erdmann, Regine Goth-Goldstein.

Poster and oral presentation at the California Breast Cancer Research Symposium, 3/02, Oakland CA, 'Metabolism of Environmental Chemicals as Breast cancer risk' by Regine

Goth-Goldstein, Christine A. Erdmann, Marion L. Russell.

Conclusions

Because of the potential important role of CYP1B1 in the activation of environmental and endogenous compounds to carcinogenic intermediates, it was hypothesized that high CYP1B1 expression could represent a risk factor for breast cancer. This is the first study to measure expression of *CYP1B1* in a collection of non-tumor breast tissue from mastectomy patients and from reduction mammoplasties, to estimate the interindividual variation of CYP1B1 levels and to compare expression in breast cancer patients and healthy individuals. We found a large interindividual variation in *CYP1B1* expression. CYP1B1 transcript levels were 2-7 fold higher than CYP1A1 in most samples indicating that CYP1B1 is an important PAH-metabolizing enzyme in the breast. *CYP1B1* expression was higher in the breast cancer group compared to the control group and the difference was statistically significant. *CYP1A1* and *CYP1B1* genotype variants were determined, but there was no difference in a certain genotype between cases and controls. CYP1B1 could play a role in breast cancer etiology by activating either environmental chemicals or endogenous substrates (estradiol) to carcinogens.

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List of personnel receiving pay from the research effort

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Marion Russell, Senior Research Associate

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Appendix Cover Sheet
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1. **Publication in Carcinogenesis**

2. **Manuscript submitted for publication to Molecular Carcinogenesis**

Carcinogenesis vol.21 no.11 pp.2119–2122, 2000

Interindividual variation in *CYP1A1* expression in breast tissue and the role of genetic polymorphism

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The cytochrome P4501A1 (CYP1A1) enzyme is regulated at the transcriptional level and its expression is influenced by genetic factors, polymorphisms in the structural and regulatory genes, and by environmental factors such as exposure to polycyclic aromatic hydrocarbons (PAHs). To investigate the role of CYP1A1 in breast cancer, we studied *CYP1A1* expression in breast tissue, thereby taking all possible modifying factors into account. We measured *CYP1A1* expression in 58 non-tumor breast tissue specimens from both breast cancer patients (n = 26) and cancer-free individuals (n = 32) using a newly developed reverse transcription–polymerase chain reaction assay. *CYP1A1* expression varied between specimens ~400-fold and was independent of age. *CYP1A1* expression was somewhat higher in tissue from breast cancer patients than in that from cancer-free individuals, but this difference was not statistically significant. Analysis for *CYP1A1* genetic polymorphisms revealed eight variants, seven in the cancer-free group and one in the patient group. The variant genotype was not a good predictor of expression level. We conclude that high *CYP1A1* expression could be a risk factor for breast cancer and that the known *CYP1A1* polymorphisms are not good predictors of *CYP1A1* expression.

Polycyclic aromatic hydrocarbons (PAHs), a class of chemicals that includes potent carcinogens, could have a role in breast cancer because they accumulate in breast adipose tissue (1) and because normal human mammary cells in culture activate PAHs efficiently (2). PAH–DNA adduct levels have been found to be significantly higher in normal breast tissue of breast cancer patients than in that of non-cancer controls (3). The mutational spectrum in the p53 gene in breast tumors resembles that of lung cancers where there is a well-established role for environmental agents, such as tobacco smoke (4). The major metabolic pathway for ingested or inhaled PAHs to water-soluble derivatives is oxidative activation by CYP1A1 followed by detoxification by phase II enzymes. There is evidence supporting a role of CYP1A1 in breast cancer from recent animal experiments: using a rat model to identify loci that control breast cancer susceptibility, one of the four loci mapped to *CYP1A1* or a nearby locus (5).

Abbreviations: CYP1A1, cytochrome P4501A1; PAHs, polycyclic aromatic hydrocarbons.

Interindividual variation in carcinogen metabolism has been recognized as a determinant of susceptibility to various cancers (6). Genetic polymorphism is one potential source of variation. For *CYP1A1*, four genetic polymorphisms consisting of single base changes have been described (7); two of them have been studied extensively as genetic biomarkers of susceptibility to various cancers (6), including breast cancer (8). The first described variant, *CYP1A1*2*, is located in the 3′ non-coding region of the *CYP1A1* gene and introduces an *Msp*I restriction endonuclease site (9,10). The second variant, *CYP1A1*3*, is strictly linked to *CYP1A1*2* (7) and consists of an A→G transition in exon 7 that results in an amino acid substitution of Val^{462} to Ile^{462} (11). Several studies have suggested that this genotype increases susceptibility to various cancers, but the biochemical basis is unclear. It has been assumed that the *CYP1A1*2* and *CYP1A1*3* alleles lead to higher inducibility. Expression of *CYP1A1* is regulated by the aryl hydrocarbon receptor, together with several other regulatory proteins. Increased transcription of the *CYP1A1* gene reflects induction of the enzyme (12). *CYP1A1* expression can be induced by exposure to PAHs and organochlorines (13). Besides environmental factors, genetic factors can modify *CYP1A1* expression; these include the genotype of the structural gene and the genotype of regulatory genes, including the aryl hydrocarbon receptor. Therefore determining the amount of transcript or the actual level of the enzyme captures the influence of all potentially modifying factors and is a more sensitive tool than the analysis of the genotype of a single gene.

We have examined *CYP1A1* expression as a possible breast cancer risk factor by comparing *CYP1A1* expression in non-tumor breast tissue from 27 breast cancer cases and 32 cancer-free individuals. Although we did not measure CYP1A1 protein levels or CYP1A1 enzyme activity, mRNA levels and enzyme activities are known to be closely related (14,15). The case specimens were derived from 22 mastectomies (peripheral non-tumor tissue) and five contralateral to carcinomatous breast. The control specimens were obtained from 32 reduction mammoplasties. Tissue specimens were dissected and isolated from adipose and connective tissue, so that only epithelial material was stored frozen as organoids (16). The pathological diagnosis of the excised tumors was intraductal carcinomas for two cases and infiltrating ductal carcinoma for the other 20 cases. In two of the 22 cases, metastasis to axillary lymph nodes was observed, indicating more advanced disease. Samples were collected without respect to age and race. Only the age and disease status of the specimen donors are known. No information is available on donors' race, lifestyle, smoking habits or other potential confounding factors. Individuals undergoing reduction mammoplasty ranged in age from 15 to 68 years, and mastectomy patients ranged in age from 30 to 87 years.

To determine *CYP1A1* transcript levels, we developed a reverse transcription–polymerase chain reaction (RT–PCR) assay that determines *CYP1A1* expression relative to the

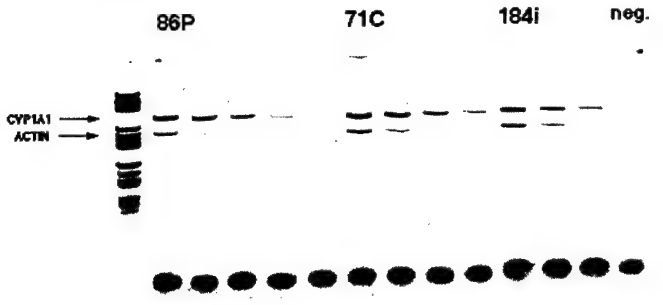

Fig. 1. Polyacrylamide gel of *CYP1A1* and β-actin PCR products for three specimens. The cDNA from each specimen was diluted serially five-fold and several of these dilutions were amplified for each specimen. Lane 1, molecular weight standard; lanes 2–6, specimen 86P peripheral to tumor; lanes 7–10, specimen 71C contralateral; lanes 11–13, 184 cells that were included in each reaction as control to test for interexperimental variation; lane 14, negative control.

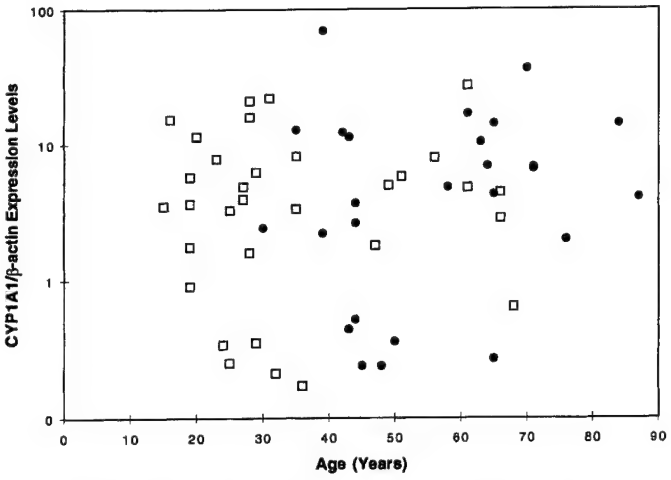

Fig. 2. *CYP1A1*:β-actin ratio as function of age of specimen donors; □ represent values of reduction mammoplasty controls and ● represent values of breast cancer cases.

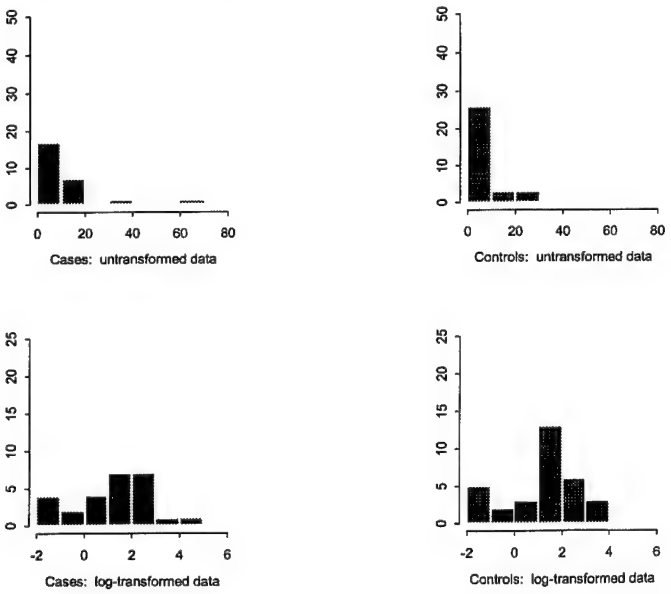

Fig. 3. Distribution of *CYP1A1* expression levels among breast cancer cases and reduction mammoplasty controls. The upper two histograms show the untransformed data. The bottom two histograms show the log-transformed data.

constantly expressed β-actin gene, thus controlling for varying sample sizes and RNA yield. Previously published primers designed to span an intron (thus excluding amplification of any contaminating genomic DNA) were used and generated products of 320 bp for CYP1A1 and 273 bp for β-actin (17,18). PCR conditions and cycle numbers were optimized separately for each target sequence to ensure that the reaction was in the linear phase of product accumulation. A five-fold serial dilution of cDNA was amplified in separate reactions for CYP1A1 and β-actin. After amplification, the products were mixed together before electrophoresis on a 10% native polyacrylamide gel. The gel was stained with SYBR Gold nucleic acid stain and scanned on a Molecular Dynamics STORM 860 optical scanner. The fluorescent signal for each band was quantified using ImageQuant software (Figure 1). We found that this assay for *CYP1A1* expression is sensitive, reproducible and has a broad dynamic range. *CYP1A1* expression was measured in 59 non-tumor breast tissues from individuals with breast cancer (n = 27) and from cancer-free individuals (n = 32). Only one of the 59 samples did not have amplifiable RNA. *CYP1A1* quantification was repeated in a blinded assay for 20% of samples. The correlation between the original measurements and the respective repeats was 0.9878, indicating that the assay is highly reproducible. In experiments with human mammary epithelial cells in culture, we found that the amount of β-actin transcript was independent of benzo[*a*]pyrene exposure, whereas *CYP1A1* transcript levels increase in proportion to the dose (data not shown). In the present study, β-actin transcript levels in the 58 specimens could be evaluated from one of the first two dilutions of the cDNA. In contrast, the whole range of dilutions was needed to determine the *CYP1A1* transcript levels in all specimens, indicating the large variations between individuals in *CYP1A1* expression. The *CYP1A1*:β-actin transcript ratio varied between the lowest value of 0.17 to the highest value of 70, a >400-fold range. As seen in Figure 2, individuals in the control group were younger than those in the case group, but *CYP1A1* expression did not change with the age of the donors. The correlation coefficient for the *CYP1A1*:β-actin transcript ratio and age was –0.0357 for cancer patients and 0.0434 for controls, constituting persuasive evidence that *CYP1A1* level and age are not correlated. The lack of a correlation with age indicates that the reduction in estrogen levels experienced with menopause does not influence the *CYP1A1* level, even though an interaction between the aryl hydrocarbon receptor and the estrogen receptor pathways has been observed in several systems (13).

CYP1A1 expression, represented by the *CYP1A1*:β-actin transcript ratio, differed between groups: The arithmetic mean of the *CYP1A1*:β-actin ratio was 9.55 (SD = 14.66) in specimens from breast cancer patients and 6.31 (SD = 6.91) in specimens from cancer-free individuals. This difference was not statistically significant (in a two-tailed t-test, t was –1.11 and P 0.27) in the small sample studied. Comparing the distribution of *CYP1A1*:β-actin values, a fairly log-normal distribution of values is seen for cases and controls (Figure 3). The geometric mean of the *CYP1A1*:β-actin ratio was 3.70 (SD = 4.90) in cases and 3.15 (SD = 4.05) in controls.

The large variation between individuals in *CYP1A1* expression might be explained by unmeasured environmental or lifestyle factors, such as smoking, which is known to induce *CYP1A1* expression. *CYP1A1* expression is increased in lung

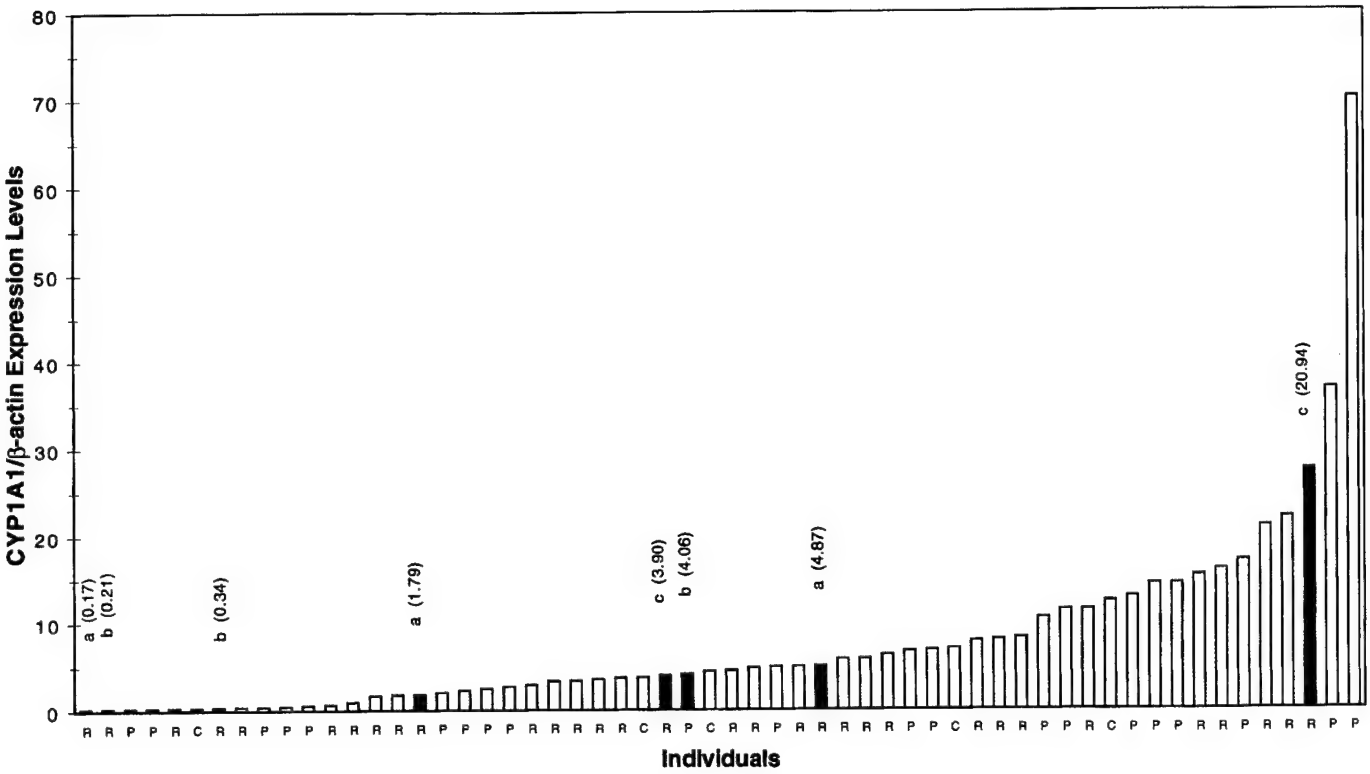

Fig. 4. *CYP1A1*:β-actin ratio ranked for all individuals. Open bars represent the *CYP1A1*1* (wild-type) genotype. Solid bars represent *CYP1A1* polymorphic variants of the following categories: (a) *CYP1A1*2* heterozygotes; (b) *CYP1A1*2/CYP1A1*1* heterozygotes; (c) *CYP1A1*2* homozygotes. *CYP1A1*:β-actin ratios are given in parentheses for the polymorphic variants. The origin of each tissue specimen is given below the bar, R, reduction mammoplasty; P, peripheral to carcinoma; C, contralateral.

tissue of patients with tobacco-induced lung cancer (19). Others have reported variation in *CYP1A1* expression in lung tissue (15,20,21), including a recent report that found that *CYP1A1* expression in females was more than twice that in males (22).

The *CYP1A1*2* and *CYP1A1*3* alleles have been associated with a phenotype of high gene induction in response to PAHs (11). To investigate to what extent the *CYP1A1* genotype modifies *CYP1A1* expression, the *CYP1A1* genotype of all specimens was determined using PCR/restriction fragment length polymorphism analysis according to published procedures (7). A total of eight *CYP1A1*2* and *CYP1A1*3* alleles in 58 samples were detected: three *CYP1A1*2* heterozygotes, three *CYP1A1*2/CYP1A1*1* heterozygotes and two *CYP1A1*2* homozygotes. The case group had only one *CYP1A1*2/CYP1A1*1* heterozygote while the control group had seven variants. When all *CYP1A1* values are ranked (Figure 4), the *CYP1A1* variants are distributed between the lowest and highest expression values. All heterozygous variants and the one homozygous *CYP1A1*2* variant have *CYP1A1* values below the mean *CYP1A1* values. Only one homozygous *CYP1A1*2* variant was among the five specimens with the highest *CYP1A1* expression values, indicating that the polymorphism has at most a minor role in determining *CYP1A1* expression.

The *CYP1A1*2* variant is located in the non-coding region of the gene, suggesting that the *CYP1A1*2* polymorphism alters the inducibility of *CYP1A1*. The *CYP1A1*3* variant is located in exon 7, which codes for the heme-binding region. A change in amino acids in this region could possibly result in a change in enzyme activity. An earlier study reported a 50% higher enzyme activity (11). However, using purified human recombinant *CYP1A1*1* and *CYP1A1*2*, a more recent study did not find different benzo[*a*]pyrene activation (23). Another study reported no difference in the kinetics of the CYP1A1 polymorphic variants (24). Therefore, any change in CYP1A1 level in *CYP1A1*3* seems to be the result of strict linkage to *CYP1A1*2* polymorphism (7), which presumably alters the inducibility of the enzyme. Our data suggest that *CYP1A1*2* polymorphism has a minor, if any, role in modifying *CYP1A1* expression (Figure 4). If individuals with the *CYP1A1* variant genotype were exposed to much lower levels of PAHs than individuals with the wild-type genotype, the impact of genotype on expression might be masked. In an earlier study, human mammary epithelial cells derived from 18 individuals were treated with benzo[*a*]pyrene and DNA adducts quantified (2). Among the strains examined were six derived from donors tested here for *CYP1A1* expression and *CYP1A1* genotype, including the two homozygous *CYP1A1*2* and one of the heterozygous *CYP1A1*2* variants identified here. Contrary to expectations, the two homozygous *CYP1A1*2* alleles had the lowest amount of adducts, indicating that the *CYP1A1*2* genotype did not increase DNA adduct formation. Besides activating xenobiotics, CYP1A1 also metabolizes 17 β-estradiol to the less active 2-hydroxy estradiol (25). A recent study suggests that *CYP1A1*2* may be a marker of altered estradiol metabolism and of increased susceptibility to estrogen-related breast cancer in African-Americans (26).

In conclusion, this study shows that breast tissue expresses a considerable range of *CYP1A1* levels independent of age and genotype, reinforcing the importance of evaluating both genotype and phenotype. Although the results are not statistically significant in the small unselected specimen groups avail-

able, they suggest that increased PAH activation by *CYP1A1* might play a role in initiation of breast cancer. Larger sample sizes will be required to corroborate these suggestive findings.

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Cytochrome P4501B1 expression in normal breast tissue [2]

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Abbreviations

B[*a*]P, benzo[*a*]pyrene; bp, base pair; CYP1A1, cytochrome P4501A1; CYP1B1, cytochrome P4501B1; HMEC, human mammary epithial cells; met, metastasis; ln, lymphnode; PAHs, polycyclic aromatic hydrocarbons; SD, standard deviation.

Running Title: CYP1B1 Expression in Breast Tissue

Keywords: CYP1B1, interindividual variation, breast cancer

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are metabolically activated to ultimate carcinogens by the cytochrome P450 isozymes CYP1A1 and CYP1B1. High levels of these enzymes may increase DNA adduct formation and cancer initiation. We investigated whether expression of *CYP1B1* in breast tissue compared with *CYP1A1* is a risk factor for breast cancer. Expression of *CYP1B1* and *CYP1A1* was measured in a collection of histologically normal breast tissue specimens from breast cancer patients and from cancer-free individuals. Using a semiquantitative RT-PCR assay, *CYP1B1* and *CYP1A1* expression levels relative to the constantly expressed β-actin gene were determined. In the study sample of 75 non-tumor epithelial breast specimens, we found about 300-fold and 1000-fold interindividual variation in expression for *CYP1B1* and *CYP1A1*, respectively. For most samples, CYP1B1 transcript levels were 2-7 times higher than CYP1A1, suggesting that CYP1B1 plays a major role in activation of PAHs in the breast. The mean CYP1B1 transcript level in normal breast tissue was 70% higher in mastectomy patients compared with cancer-free individuals ($p = 0.0473$). These data suggest that CYP1B1 may play a role in breast cancer etiology.

Abbreviations

B[*a*]P, benzo[*a*]pyrene; bp, base pair; CYP1A1, cytochrome P4501A1; CYP1B1, cytochrome P4501B1; HMEC, human mammary epithial cells; met, metastasis; ln, lymphnode; PAHs, polycyclic aromatic hydrocarbons; SD, standard deviation.

Running Title: CYP1B1 Expression in Breast Tissue

Introduction

Ingested or inhaled PAHs are metabolized to water-soluble derivatives through oxidative activation primarily by the cytochrome P450 enzymes CYP1A1 and CYP1B1. CYP1A1 has been studied extensively for over 25 years. Much less is known about the more recently discovered CYP1B1, which is related to CYP1A1 in DNA sequence and substrate specificity(1). The CYP1B1 enzyme is involved in the activation of a number of lipophilic environmental carcinogens, including PAHs and aromatic amines (2). CYP1B1 also has an endocrine regulatory role; it hydroxylates 17 ß-estradiol at the C-4 position to the potentially carcinogenic 4-hydroxyestradiol (3,4), which imparts estrogenic activity (5). In addition, CYP1B1 has an as yet undefined endogenous role, as truncating *CYP1B1* mutations have been linked to primary congenital glaucoma (6). CYP1B1 shows tissue-specific expression that is distinct from CYP1A1. CYP1B1 is expressed at minimal levels in liver, the major site for metabolism of xenobiotic compounds, but is constitutively expressed in some extrahepatic tissues, including steroid-responsive tissues such as breast, uterus, and prostate (2).

Recent evidence suggests that CYP1B1 might be more important than CYP1A1 in PAH carcinogenesis. Experiments with recombinant human enzymes showed that CYP1B1 catalyzes oxidation of benzo[a]pyrene (B[a]P) to the diolepoxide ten times more efficiently than CYP1A1 (7). In 7,12-dimethylbenz(a)anthracene-treated mice, the rate of lymphomas in CYP1B1 null mice was one tenth the rate in wild-type mice (8). These data demonstrate the importance of extrahepatic metabolism in determining susceptibility

to PAHs and establish CYP1B1 as an important contributor to PAH carcinogenesis in tissues where CYP1B1, in contrast to CYP1A1, is constitutively expressed.

Because the CYP1B1 and CYP1A1 enzymes activate potential carcinogens, they are thought to play an important role in tumor development (9). In a previous analysis of *CYP1A1* expression levels in a collection of non-tumor breast specimens from breast cancer cases and controls, no statistically significant differences were found between cases and controls (10). In this analysis, we examine expression of *CYP1B1* in breast tissue of cases and controls to gain insight into its potential role in breast cancer. Expression of *CYP1B1* was higher than *CYP1A1* in over 80% of samples and notably higher in specimens from cases compared with those from controls.

Materials and methods

Materials. Primers for PCR were synthesized by Life Technologies (Rockville, MD). Reverse transcriptase, $(oligo\ dT)_{16}$, dNTPs, and Rnase inhibitor were purchased from Promega (Madison, WI). Tri Reagent, agarose, and Taq DNA polymerase were obtained from Sigma Chemical Co. (St. Louis, MO). Electrophoresis chemicals were purchased from Bio-Rad (Richmond, CA). Molecular weight standards were obtained from New England BioLabs (Beverly, MA). SYBR Gold nucleic acid gel stain was purchased from Molecular Probes (Eugene, OR). All other reagents were purchased from Sigma Chemical Co. and were molecular biology grade when available.

Specimens. Two collections of non-tumor breast tissue from breast cancer patients and cancer-free individuals were used; one was established by M. Stampfer (11); and the

other was obtained from Aeron Biotechnology and was collected using the Stampfer protocol. These tissue banks contain specimens from reduction mammoplasties and mastectomies, including tumor and peripheral non-tumor tissue. In a few cases, contralateral tissue and lymph node metastatic tissue was available. The specimens were dissected and isolated from adipose and connective tissue, so that only epithelial material was stored frozen as organoids. The pathological diagnosis of the excised tumors was intraductal carcinomas for 2 cases and infiltrating ductal carcinoma for the other 34 cases. In 5 of the 36 cases, metastasis to axillary lymph nodes was observed indicating more advanced disease. Samples were collected without respect to age or race. Only the age and disease status of the specimen donors are known. No information is available on donors' race, lifestyle, smoking habits, or other potential confounding factors. Individuals undergoing reduction mammoplasty ranged in age from 15 to 73 years. The age of mastectomy patients ranged from 30 to 87 years.

Cell culture. cDNA from human mammary epithelial cells (HMEC) in culture was included in each PCR reaction as a positive control. Primary cultures of reduction mammoplasty tissue specimen 184 were grown in the serum-free medium MCDB170 as described by Stampfer (11). Ten 100-mm culture dishes with proliferating HMEC 184 cells (passage12) were treated with 10 μM B[*a*]P for 20 hours, then washed twice with cold PBS before lysing with Tri Reagent as described below.

RNA, DNA preparation, and cDNA synthesis. Total RNA and genomic DNA were simultaneously isolated from the specimens with TRI Reagent following the procedure recommended by the manufacturer. Before reverse transcription, RNA was heated at 72° C for two minutes then chilled on ice to remove secondary structure. Two μg of total

RNA was diluted to a final volume of 25 μl with 0.1 μg (oligo dT)$_{16}$, 0.5 mM dNTPs, 10 units of RNase inhibitor, 100 units of Moloney murine leukemia virus reverse transcriptase, 10 mM Tris (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, and 5 mM dithiothreitol. The samples were incubated at 37°C for 1 hour followed by heat inactivation at 98°C for 3 minutes. The cDNA was stored as several small aliquots at -80°C.

Measurement of CYP1B1 and CYP1A1 expression by semiquantitative RT-PCR.

CYP1B1 and *CYP1A1* expression were measured in parallel by a semiquantitative method developed in our lab (10; Russell *et al.*, in preparation). cDNA obtained from the breast epithelial cells was serially diluted, then amplified three times: first with CYP1B1 primers (12), then with CYP1A1 primers (13), and finally with β-actin primers (14). All primer sets are designed to span an intron, thus excluding amplification of any contaminating genomic DNA and generated products of 545 bp for CYP1B1, 320 bp for CYP1A1, and 273 bp for β-actin. As PCR is quantitative only in the exponential phase of product accumulation, PCR conditions and cycle numbers for each target were optimized separately for each primer pair. The cycle numbers for each target were chosen to give appropriate sensitivity while avoiding PCR plateau phase. The CYP1B1 RT-PCR assay was optimized to give the same amplification efficiency as the CYP1A1 reaction. All necessary procedures were used to avoid PCR product contamination in the set-up of the reaction mixtures (15). Each 50μl PCR contains 5µl of cDNA dilution, 10 mM Tris (pH 8.3), 50 mM KCl, 0.2 mM each dNTP and 1.5 units of Taq polymerase. All targets are amplifed on the same day in an MJ Research model PTC-100 thermal cycler using "hot start" conditions. β-actin was amplified using 25 pmoles of each primer, 2 mM $MgCl_2$, and 18 cycles as follows: 30 sec. at 96°C, 30 sec. at 58°C and 30 sec. at 72°C for 2 cycles

followed by 30 sec. at 94°C, 45 sec. at 56°C and 1 min at 72°C for 16 cycles followed by 10 min. at 72°C. CYP1A1 was amplified using 50 pmoles of each primer, 6 mM $MgCl_2$, for 20 cycles. The CYP1A1 product obtained by this procedure has been confirmed by sequencing. CYP1B1 was amplified using 40 pmol of each primer and 2mM $MgCl_2$ by the same thermal cycling conditions as CYP1A1. Thermal cycler parameters for CYP1A1 and CYP1B1 were 30 sec. at 96°C, 30 sec. at 63°C with a 1°C per cycle decrease, and 30 sec. at 72°C for 7 cycles followed by 30 sec. at 94°C, 45 sec. at 56°C and 1 min. at 72°C for 20 cycles followed by 10 min. at 72°C. Product was stored at 4°C overnight. For each set of samples analyzed, cDNA obtained from 184 HMEC (induced with B[a]P) was included as a positive control and sterile water was included as a negative control.

After amplification, the products were mixed together before electrophoresis on a 10% native polyacrylamide gel. The gel was stained for 10 minutes using SYBR Gold nucleic acid stain (Figure 1). Gels were scanned without destaining on a Molecular Dynamics STORM 860 laser scanner. The fluorescent signal for each band was quantitated using ImageQuant software after background correction included in the software. CYP1A1 and CYPIB1 levels are expressed as a ratio to β-actin values.

Data analysis. The linear range of the Storm 860 scanner and the SYBR Gold signal intensity were determined by analysis of a dilution series of a mass standard (data not shown). All of the fluorescent signals generated by the PCR products remained in this range or they were discarded and the specimen was re-amplified using a lower cDNA dilution. Specimens were processed in groups of ten. Each group included a positive and a negative control. Four cDNA dilutions (of a 4-fold dilution series) were amplified for

each specimen. Each run was considered valid if the negative control showed no bands at 273 and 320 and 545 bp and if the positive contol showed bands at 273 bp and 320 and 545 bp at a predetermined acceptable signal intensity. For each specimen, the fluorescent signal of the cDNA dilution was accepted for analysis if the signal was at least twice the signal of the negative control and if the signal was not in plateau. PCR plateau was determined by examining the signal increase of the dilution series. If the signal intensity no longer increased by 4-fold, that dilution was not used in the analysis. After the signal for a dilution was accepted to be valid, the CYP1B1 level of each specimen was expressed as:

CYP1B1 level = (CYP1B1 fluor. signal x DF)/(β-actin fluor. signal x DF),

where DF is the dilution factor. Samples analyzed in different groups of 10 were compared after assuring that values of the positive control were in the previously measured range. CYP1A1 levels were determined in a similar fashion. After all the specimens were analyzed, 15% of the cDNA was re-amplifed and the CYP1B1 and CYP1A1 levels were found to repeat within two-fold.

The linear relationship between age and CYP1B1 level was evaluated using Pearson's product moment correlation coefficient. Descriptive analyses of CYP1B1 levels by case-control status and age group (< 50 years versus ≥ 50 years) included calculation of arithemetic means, geometric means, standard deviations, and medians. Means were compared using the two-tailed Student's t-test.

Results

The control group consisted of 39 reduction mammoplasty specimens. The case group

consisted of 32 peripheral non-tumor tissue specimens obtained from mastectomy patients and 4 tissue specimens contralateral to the tumor.

The reliability of the semiquantitative assay to detect *CYP1B1* expression was tested by measuring expression of these genes in triplicate samples from the same individual (separately frozen down). The results showed a good agreement between the three samples with less than 3% variance in CYP1B1 expression. It was also determined how similar *CYP1B1* expression is in specimens from left and right breast to address the question of local variation of gene expression in breast tissue. The tissue specimens from left and right breast had been processed as separate samples. For three pairs tested, CYP1B1 transcript levels varied by less than 15% among each pair (CYP1B1/β-actin in breast tissue from left versus right: 0.97, 1.18; 3.12, 2.69; 1.17, 1.00). Comparing *CYP1B1* expression in breast cells and skin cells of one individual, a 30-fold difference in CYP1B1 transcript levels was found (CYP1B1/β-actin in breast tissue 29.2 in skin 0.92), indicating a large variation in *CYP1B1* expression between different tissues in one individual.

CYP1B1 transcript levels versus CYP1A1 transcript levels. In HMEC 184 (passage 12 cells treated with 10 μM B[*a*]P for 20 hours), which are included as a positive control, CYP1B1/β-actin ranged from 5.92 - 12.61 (mean = 8.67) in the 12 measurements, CYP1A1/β-actin ranged from 0.81- 1.79 (mean = 1.24). The CYP1B1 to CYP1A1 ratio was 7.0. In untreated HMEC 184 cells CYP1B1/β-actin was almost 50 times higher than CYP1A1/β-actin (data not shown).

In breast tissue specimens from cases and controls, there was a more than 300-fold variation between individuals in the CYP1B1/β-actin ratio (range 0.43 to 132.50), and a

more than 1000-fold variation in the CYP1A1/β-actin ratio (range 0.06 to 66.81). As seen in Figure 2, *CYP1B1* was expressed at lower levels than *CYP1A1* in only one specimen, at similar levels as CYP1A1 in 13 specimens (ratio 0.8-1.4), and at higher levels than *CYP1A1* (ratio 2 - 82) in the remaining 61 specimens (81% of the sample). The mean CYP1B1 to CYP1A1 ratio was 7.98 and the median ratio was 3.9. The difference in the mean ratio between cases (mean = 7.84, SD = 13.01) and controls (mean = 8.11, SD = 10.58) was not statistically significant (p = 0.9271). Seven specimen donors (3 cases and 4 controls) had a CYP1B1 : CYP1A1 ratio $\geq$ 20; all were under 50 years of age.

***CYP1B1* expression in cases versus controls and as function of age.** The difference in CYP1A1 levels between cases and controls was not statistically different (10, and Table I). When *CYP1B1* expression in cases and controls was compared (Table I), the mean CYP1B1 transcript level was about 70% higher in cases than in controls (p = 0.0473). This difference was attenuated when evaluating the log transformed data (Table I). As shown in Figure 3, the range of *CYP1B1* expression was much greater in cases than in controls.

CYP1A1 expression in breast tissue has been found previously to be independent of age (10). *CYP1B1* expression does not appear to be linearly related to age (r = 0.071, p = 0.5549). However, when the data were split into two age groups (< 50 and $\geq$ 50), which roughly correspond to menopausal status, the range of the CYP1B1 values was noticeably more restricted in controls over 50 years of age (Figure 4), possibly indicating that endogenous estrogen levels might influence *CYP1B1* expression.

***CYP1B1* and *CYP1A1* expression in non-tumor versus tumor tissue.** For 5 cases, non-tumor tissue, tumor tissue, and/or metastasis to the lymph nodes from the same individual were available. We compared *CYP1B1* expression in tumor tissue versus normal tissue to evaluate whether higher CYP1B1 levels might be related to the disease process. The results were ambiguous (Table II). For one individual, non-tumor tissue had higher *CYP1B1* expression than tumor tissue. For 3 individuals, expression was higher in tumor than non-tumor tissue. In one individual, where 2 different tumors and 2 metastases to the lymph node were available, *CYP1B1* expression in nontumor tissue, 1 tumor, and 1 metastasis was comparable, but in 1 tumor and the other metastasis, *CYP1B1* expression was increased 3-fold and 9-fold, respectively. CYP1A1 expression varied less between non-tumor and tumor tissue.

Discussion

Interindividual variation in carcinogen metabolism is a determinant of susceptibility to various cancers (16). Variability in carcinogen metabolism can be due to the level of the enzymes or to the catalytic activity of the enzymes. Genetic polymorphisms can alter the catalytic properties of an enzyme. Two common genetic polymorphisms consisting of single base changes have been described for *CYP1B1* (17). A change in catalytic activity towards steroid hormones has been observed in these *CYP1B1* variants (18, 19). The role of these polymorphisms as risk factors for cancer is not yet clear. Two studies on *CYP1B1* polymorphism and breast cancer risk conducted in different ethnic groups gave conflicting results (17,20). A recent study found that the *CYP1B1* variant with increased activity for 4-hydroxyestradiol formation is a susceptibility factor for ovarian cancer (21).

Whereas a polymorphism represents a lifelong rigid category with a defined effect, the expression level of a gene is quite variable; measuring expression provides a more differentiated response. Several enzymes involved in xenobiotic metabolism are expressed in breast (22-24). Our primary goal in this study was to analyze *CYP1B1* expression in normal breast tissue and determine whether it could have a role in breast cancer development. *CYP1A1* expression in response to PAHs and organochlorines and its regulation by the Ah receptor has been studied extensively. It is generally accepted that CYP1A1 transcript levels mirror enzyme levels (25). CYP1B1 mRNA levels also have been found to correlate with comparable changes in the protein levels as a result of transcriptional activation (26, 27, 12). In mouse fibroblasts, CYP1B1 levels are regulated not only by the Ah receptor, but can also involve protein stabilization (28). No such regulatory mechanism has been described in human cells. Therefore, *CYP1B1* expression is assumed to be a proxy measure for CYP1B1 protein levels.

CYP1B1 is expressed constitutively in human mammary epithelial cells in culture, whereas CYP1A1 is detected only after cellular exposure to environmental chemicals (12). We found that *CYP1B1* expression was much higher than *CYP1A1* expression in the cultured HMEC 184. For 184A1, an immortalized cell line derived from 184 after treatment with B[*a*]P the opposite was observed (29). The 184A1 cells, however, were grown in different cell culture media, and cell-specific expression depends on culture conditions (30). CYP1B1 transcript levels were higher than CYP1A1 transcript levels in more than 80% of the samples tested in our study. CYP1B1 therefore appears to be a predominant PAH-activating enzyme in the breast. *CYP1B1* expression varied more than 300-fold among the 75 specimens tested. Interindividual variation in *CYP1B1* expression

has been observed by others: 2.5-fold variation in early passage HMEC from 7 women (12); and 30-fold variation in CYP1B1 transcript levels in uncultured lymphocytes obtained from 10 individuals (31).

The Ah receptor induces expression of both *CYP1A1* and *CYP1B1*, though *CYP1B1* is induced to a lesser extend. The observed interindividual variation in *CYP1B1* and *CYP1A1* expression may be due to differentialexposure of individuals to Ah receptor ligands, such as PAHs and organochlorines. Possible environmental exposures might be through diet, smoking, or ambient air. Simultaneous high CYP1A1 and CYP1B1 transcript levels in breast tissue may be indicative of exposure to PAHs or organochlorines. In 7 specimens from cases and 3 from controls, both CYP1A1 and CYP1B1 transcript levels were above the 75% quantile. High levels of CYP1A1 and CYP1B1 have similar effects on the activation of environmental procarcinogens, both lead to increased activation. However, stereoselective differences in activation by CYP1A1 and CYP1B1 have been observed and can have important consequences for the carcinogenicity of the ultimate carcinogen formed (32).

Increased levels of CYP1A1 and CYP1B1 have a different effect on estrogen metabolism. Even though both CYP1A1 and CYP1B1 can metabolize 17 ß-estradiol, a high level of CYP1A1 will result in conversion to an inactive metabolite, 2-hydroxyestradiol, whereas a high level of CYP1B1 will lead to enhanced formation of the potentially carcinogenic 4-hydroxyestradiol. The 4-hydroxyestradiol metabolite has similar estrogenic activity as 17 ß-estradiol and can be further oxidized to a quinone that can depurinate DNA or lead to formation of reactive oxygen species (5, 33, 34)

CYP1B1 activity has been found to be higher in breast cancer tumors than in adjacent normal tissue (35). In an immunohistochemical study, CYP1B1 activity was shown to be expressed in a wide range of tumors, but was not detectable in normal tissue by the method used (36). In contrast, a recent study found strong immunohistochemical staining of the CYP1B1 protein in all breast tissues tested and the staining was both nuclear and cytoplasmic (37). We observed some variation in CYP1B1 levels of non-tumor and tumor tissue from the same individual, though no consistent pattern was apparent (Table 2). This might indicate that changes in *CYP1B1* expression are not part of the disease process, but rather a sign of the multiple, random changes occuring in tumor progression. While there was no clear linear relationship between CYP1B1 levels and age, there is possibly a relationship between CYP1B1 levels and menopausal status. In controls $\geq$ 50 years of age CYP1B1 transcript levels were all less than 30, whereas 25% cases $\geq$ 50 years of age had levels greater than 30. Caucasian breast cancer patients with the *CYP1B1* variant with increased activity for 4-hydroxyestradiol formation have a significantly higher percentage of estrogen receptor positive/progesterone receptor positive tumors (17). Increased CYP1B1 metabolism through high enzyme levels or through the high activity *CYP1B1* variant might be correlated with development of estrogen receptor positive tumors, the predominant tumor type in postmenopausal women (38). This study is limited by the small number of unselected specimens. This small sample size precluded a meaningful analysis stratified by *CYP1B1* polymorphism. A case-control study involving a larger number of individuals and collection of information about potential confounders is planned to further elucidate the relationship between *CYP1B1* genotype, expression, and breast cancer risk. Since human CYP1B1 is capable

of activating diverse xenobiotic procarcinogens and also hydroxylate 17 β-estradiol to 4-hydroxyestradiol, it could play a role in breast cancer etiology by activating either environmental chemicals or endogenous substrates to carcinogens.

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Table I: CYP expression summary statistics for cases and controls

CYP	Summary Statistic	Cases	Controls	p-value
CYP1A1	arithmetic mean (SD)	8.39* (11.57)	6.00* (8.12)	0.3005
	geometric mean (SD)	4.18 (3.82)	2.40 (5.03)	0.1111
	median	5.24	3.81	N.A.
	range	0.24 - 66.81	0.06 - 38.35	N.A.
CYP1B1	arithmetic mean (SD)	29.53(32.60)	17.48 (17.42)	0.0473
	geometric mean (SD)	15.82 (3.41)	10.18 (3.37)	0.1225
	median	16.13	10.48	N.A.
	range	1.52 - 132.5	0.43 - 79.3	N.A.

* These values reflect repeat measurements of CYP1A1 expression simultaneously with CYP1B1 expression of 56 specimens previously analyzed in ref. 10 (32 controls and 24 cases).

Table II: Comparison of CYP1B1 and CYP1A1 expression in non-tumor versus tumor tissue for five individuals

ID	Specimen	CYP1B1/β-actin (relative difference)		CYP1A1/β-actin (relative difference)	
380	peripheral/non-tumor	4.46	(1.00)	0.74	(1.00)
	tumor in left breast	11.45	(2.57)	0.77	(1.04)
	tumor in right breast	4.45	(1.00)	0.45	(0.61)
	met* to left ln*	4.83	(1.08)	0.35	(0.47)
	met* to right ln*	41.87	(9.39)	6.21	(8.39)
383	peripheral/non-tumor	10.41	(1.00)	7.56	(1.00)
	tumor	6.31	(0.61)	1.33	(0.18)
407	peripheral/non-tumor	1.65	(1.00)	0.25	(1.00)
	tumor	6.92	(4.19)	1.73	(6.92)
412	peripheral/non-tumor	15.45	(1.00)	4.45	(1.00)
	tumor	66.88	(4.33)	10.80	(2.43)
426	peripheral/non-tumor	4.60	(1.00)	5.75	(1.00)
	met* to ln*	71.40	(15.52)	9.51	(1.65)

* met – metastasis, ln – lymphnode

Figure Legend

Fig. 1: Polyacrylamide gel of quantitated CYP1B1, CYP1A1, and β-actin PCR products for 3 specimens (CYP1B1 545 bp fragment, CYP1A1 320 bp fragment, and β-actin 273 bp fragment). The cDNA from each specimen was diluted serially 4-fold and several of these dilutions were amplified for each specimen. The cDNA dilution chosen for each gene target varied in order to keep the PCR signal in the quantitative range. Lane 1, molecular weight standard; lane 2-5, lane 6-8, lane 9 -11 represent the serial dilutions of 3 different specimens; lane 12, negative control.

Fig. 2: CYP1A1/CYP1B1 to β-actin ratio in non-tumor breast tissue for all individuals ranked by increasing CYP1B1 values. Open bars represent the CYP1B1. Solid bars represent CYP1A1. The origin of each tissue is given below the bar, R, reduction mammoplasty (control); P, peripheral to carcinoma (case); C, contralateral.

Fig. 3: Distribution of *CYP1B1* expression levels in non-tumor breast tissue among breast cancer cases (solid bars) and reduction mammoplasty controls (open bars).

Fig. 4: Scatter plots of *CYP1B1* expression levels (relative to β-actin) by age for cases and controls stratified by <50 and ≥ 50 age group.

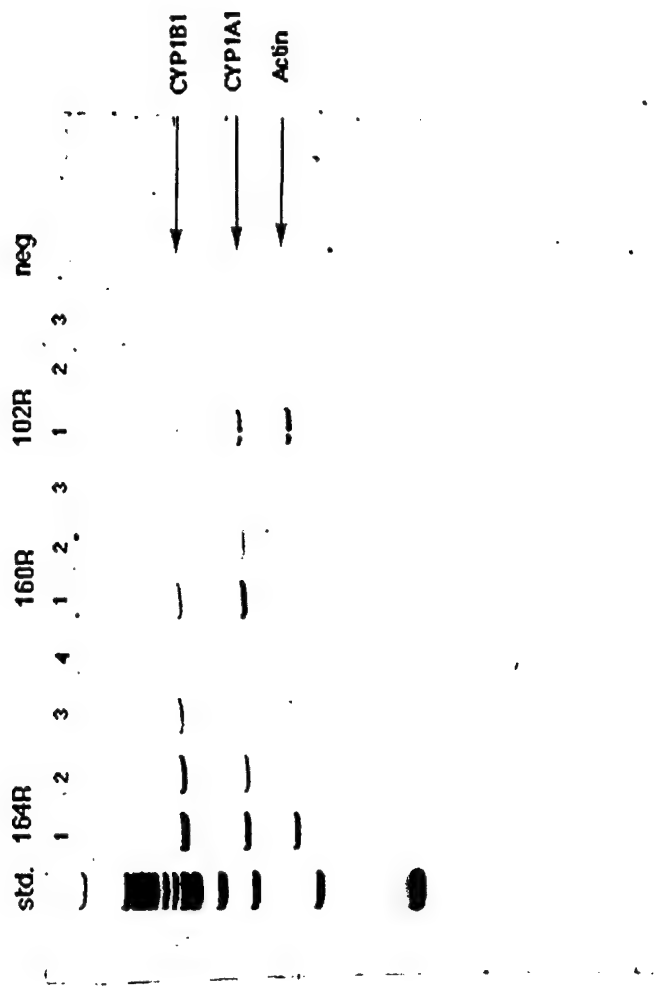
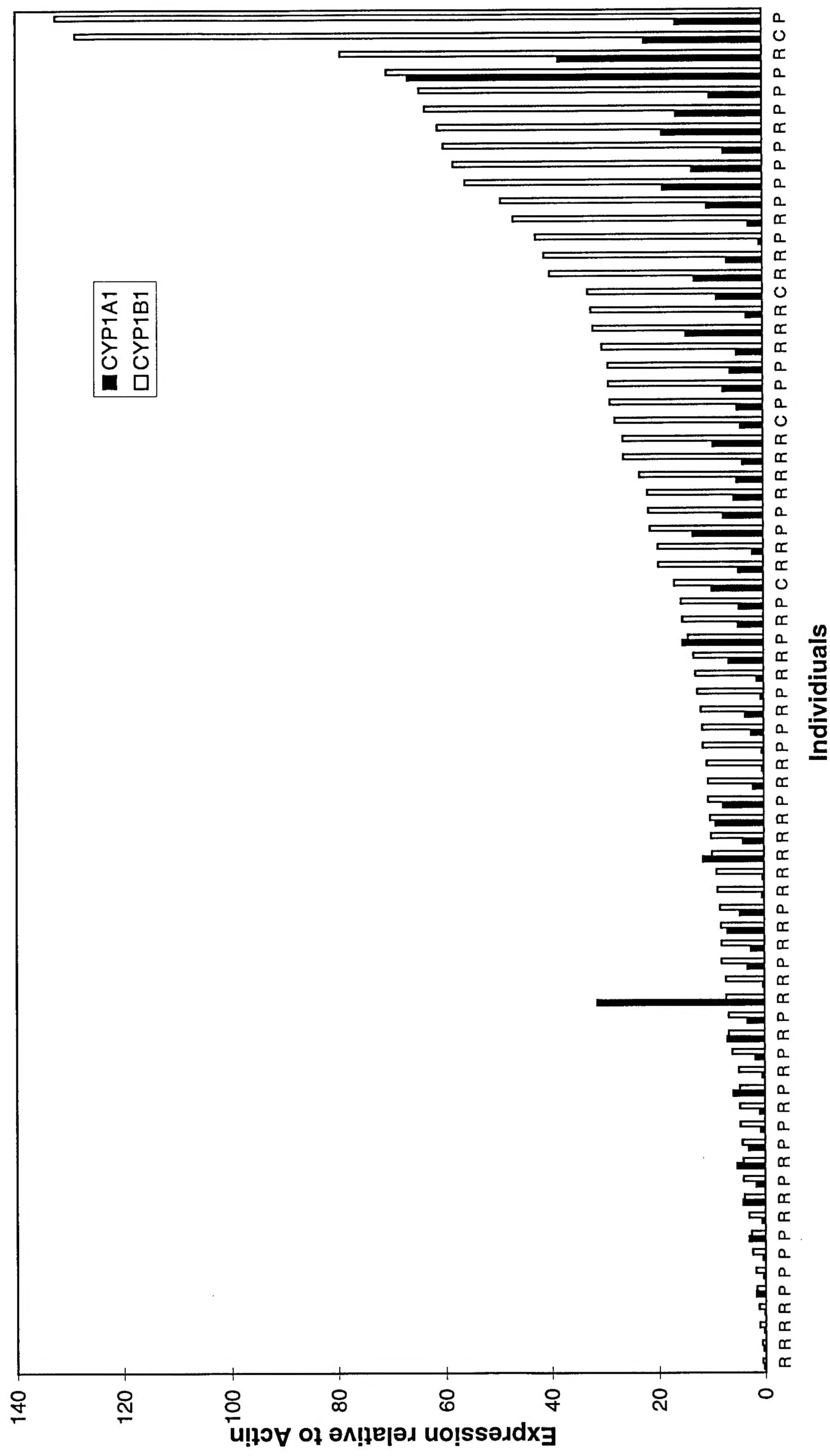


Figure 1



CYP1A1
CYP1B1
Expression relative to Actin
0
20
40
60
80
100
120
140
Individuals

Figure 2

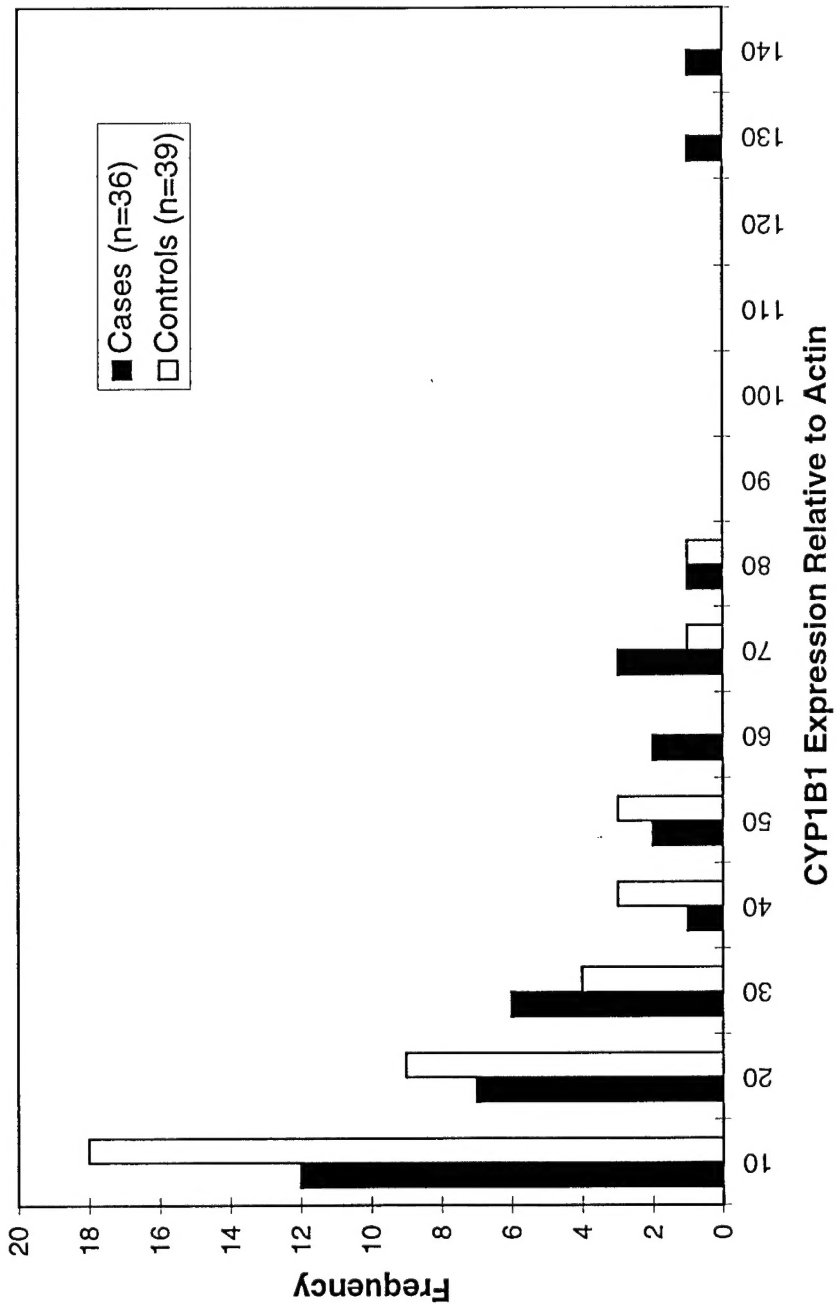
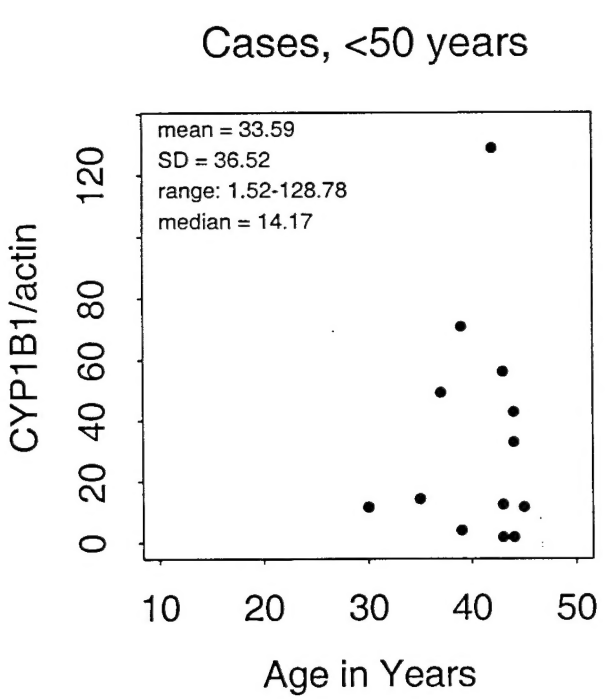
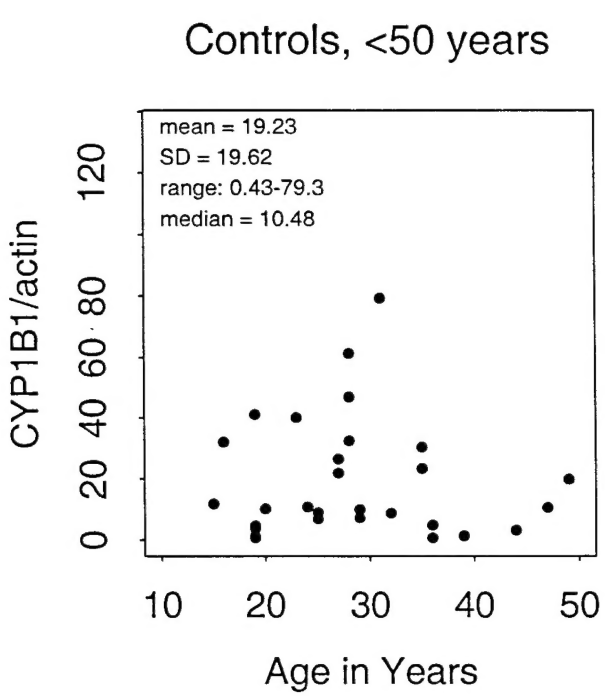
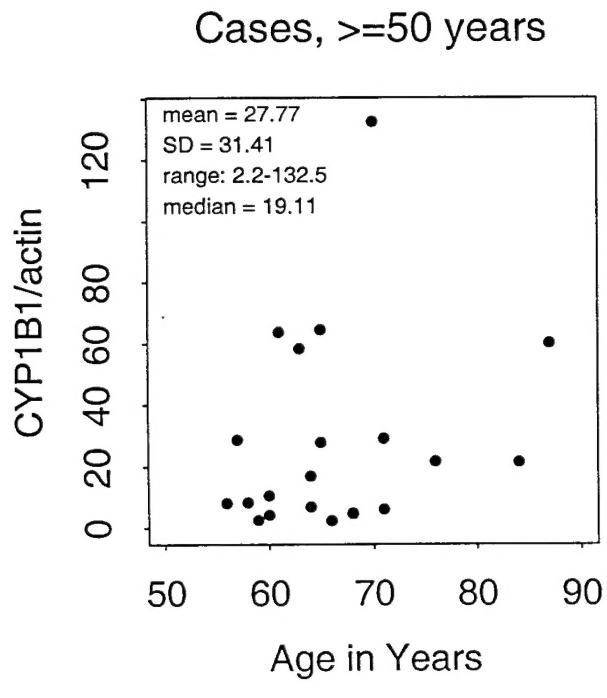

Cases (n=36)
Controls (n=39)
Frequency
0
2
4
6
8
10
12
14
16
18
20
10
20
30
40
50
60
70
80
90
100
110
120
130
140
CYP1B1 Expression Relative to Actin

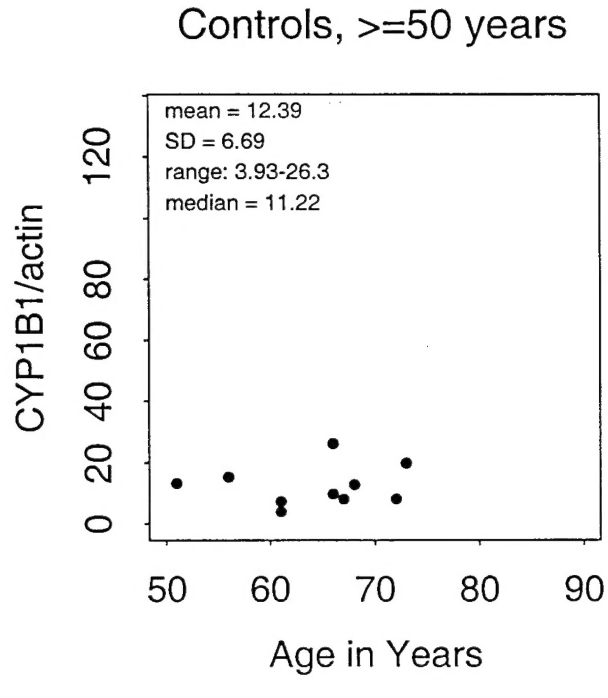
Figure 3

Figure 4


Cases, <50 years
mean = 33.59
SD = 36.52
range: 1.52-128.78
median = 14.17
CYP1B1/actin
0 20 40 60 80 120
10 20 30 40 50
Age in Years


Controls, <50 years
mean = 19.23
SD = 19.62
range: 0.43-79.3
median = 10.48
CYP1B1/actin
0 20 40 60 80 120
10 20 30 40 50
Age in Years


Cases, >=50 years
mean = 27.77
SD = 31.41
range: 2.2-132.5
median = 19.11
CYP1B1/actin
0 20 40 60 80 120
50 60 70 80 90
Age in Years


Controls, >=50 years
mean = 12.39
SD = 6.69
range: 3.93-26.3
median = 11.22
CYP1B1/actin
0 20 40 60 80 120
50 60 70 80 90
Age in Years

List of Personnel receiving pay from the Research Effort

Regine Goth-Goldstein, Principal Investigator

Marion Russell, Senior Research Associate